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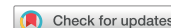


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ORIGINAL ARTICLE



The effect of *N*-acetyl cysteine and doxycycline on TNF- α -Rel-a inflammatory pathway and downstream angiogenesis factors in the cornea of rats injured by 2-chloroethyl-ethyl sulfide

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ABSTRACT

Background: Cornea injury of sulfur mustard (SM) is considered as the most devastating injuries to the eye. This study aimed to evaluate the single and combined effects of *N*-acetyl cysteine (NAC) and doxycycline on the inflammatory pathway and cornea neovascularization (CNV) in the rat model of SM-injured cornea.

Materials and methods: The right cornea of male Sprague–Dawley rats was subjected to 2-chloroethyl-ethyl sulfide (CEES). Rats were topically treated with a single and combined of 0.5% NAC and 12.5 μ g/ml doxycycline and examined at 3rd, 15th, and 21st days. The activity of three antioxidant enzymes was analyzed in the cornea of different groups. Real-time PCR was performed to measure gene expression of inflammatory factors (*tnf- α* , *rel-a* & *ccl-1*) and angiogenesis factors (*vegfa*, *mmp2,9*) in the cornea lysates. The histological and opacity assessments were also carried out.

Results: The activity of antioxidant enzymes significantly declined 3 days after the CEES damage. NAC eye drop recovered the enzyme activity on the 21st day of treatment (p -value < .05). The expression of *tnf- α* and *rel-a* genes significantly increased after CEES cornea exposure, while NAC declined their expression on the 7th and 21st days. The CNV score and angiogenesis factor expression were decreased in the long term by single and combined treatments (p -value < .05), but the infiltration of inflammatory cells was not completely amended.

Conclusion: NAC and doxycycline eye drop could improve the CNV complication. Also, NAC was an effective treatment against the inflammatory pathway involved in CEES-injured cornea.

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KEYWORDS

Cornea neovascularization; doxycycline; inflammation; *N*-acetyl cysteine; sulfur mustard



Introduction


Vesicating chemical warfare agents cause the body severe damages, particularly in respiratory, skin, and ocular systems. Sulfur mustard (SM; bis [2-chloroethyl] sulfide) has been widely used as the vesicating agent, which can induce ocular injury [1]. The symptoms such as photophobia, corneal ulceration, and lacrimation are clinically manifested the acute ocular injury following 2–6 h exposure. In the delayed phase, the ocular injury develops conjunctival scarring, corneal neovascularization (CNV), inflammation, and epithelial cornea defects that can result in progressive visual impairment [2,3]. SM can penetrate the corneal epithelium and damage cell structure by producing high levels of reactive oxygen species (ROS), reactive nitrogen species (RNS) [1].

The pro-inflammatory cytokines are the first mediators released after oxidative stress at the cornea to stimulate immune system cells [3,4]. The use of topical corticosteroids

and nonsteroidal anti-inflammatory drugs (NSAIDs) cannot efficiently recover the CNV and inflammation caused by SM-induced ocular injury. Understanding the pathological mechanisms of SM-induced ocular injury may lead to developing effective therapeutic strategies [4,5]. There is a global effort to find out appropriate therapeutic strategies for SM-induced ocular injury, however, after a century, investigations have not able to represent an approved U.S. Food and Drug Administration (FDA) countermeasures against SM-injured cornea [6].

N-Acetyl cysteine (NAC), a precursor of the amino acid cysteine with thiol agent, scavenges ROS through increasing the level of mitochondrial and cytosolic glutathione [7]. Several studies have shown that NAC has a long-term effect in preventing damage to the protein and DNA of cells exposed to the active oxidants compounds [8,9]. NAC also reduces the expression of the NF- κ B transcription factor by reducing intracellular thioredoxin [10]. Investigations have

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reported the anti-inflammatory properties of tetracycline family antibiotics in human corneal epithelial cells [11,12]. Doxycycline is a semisynthetic long-acting tetracycline with anti-inflammatory effects that are attributed to the inhibition of p38 MAPK signaling [13]. Besides, doxycycline can reduce the migration of immune cells to the cornea of the alkali-burned mice model [14].

2-Chloroethyl-ethyl sulfide (CEES) is a structural analog of SM modifying biomolecules within the cell and causes ocular injury similar to SM [6]. In this study, we evaluated the clinical and pathophysiological effects of single and combined topical treatment of NAC and doxycycline against CEES-injured cornea in rat models. During a 21-day post-exposure, molecular and intensity of oxidative stress indicating the state of inflammatory and angiogenesis factors were analyzed at 3rd, 7th, and 21st days. Also, histological data were examined to understand the possible healing effects of treatments on the injury.

Material and method

Animal models and experimental design

A total of 90 healthy male Sprague–Dawley (SD) rats, weighing from 180–220 g, were used in this study. The rats were kept at suitable conditions with 40–60% humidity, $24 \pm 2^\circ\text{C}$ temperature, 12 h of light, and 12 h of darkness. The treatment procedure was applied in accordance with the Animal use guidelines 2016 published by the Association for Research in Vision and Ophthalmology (ARVO) [15]. This experiment was approved by the Regional Research Ethics Committee of Bagiatollah Medical Sciences University (IR.BMSU.REC.1398.380). All rats were randomly divided into five groups that each group includes 18 rats: Group 1 (Healthy group): no CEES injury and right eyes were treated by normal saline drop as the vehicle-treated group; Group 2 (CEES group): right eyes were exposed to CEES at the 0 day; Group 3 (NAC + CEES group): right eyes were treated with NAC drop following CEES corneal exposure; Group 4 (Dox + CEES group): right eyes were treated with doxycycline drop following CEES corneal exposure; Group 5 (Dox + NAC + CEES group): right eyes were treated with doxycycline and NAC drop following CEES corneal exposure. The CEES corneal injury occurred on day 0. Six rats from each group were euthanized 3, 7, and 21 days after injury and sacrificed for the next experiments.

In vivo animal model of corneal SM injury

Before beginning the study, all rats received 0.3% tobramycin eye drops to prevent corneal infection [16]. At the 0 day, rats belonging to the groups 2–5 were anesthetized by 35 mg/kg body weight, ketamine hydrochloride 10% and xylazine 2% combination (Alfasan Company, Woerden, The Netherlands) to painless the corneal analgesia. The right eyes of rats were maintained open, and corneal analgesia was creating by dropping 10 μl neat CEES (24 μl of 98% CEES liquid was initially dispersed into 76 ml of 100% ethanol to produce 2 M concentration of CEES, and then was dissolved in normal

saline to produce 3 $\mu\text{mol/ml}$ CEES) (Sigma-Aldrich, St. Louis, MO, CAS no. 693-07-2) according to a similar study [17]. The CEES was placed at the center of the cornea for 45 s. Subsequently, the cornea was rapidly washed with 200 μl sterile saline solution for three times. In addition to clinical assessment. The occurrence of the corneal injury was checked with a slit lamp 4 h after CEES exposure.

The right eyes of the NAC + CEES group were treated twice daily with 1 drop of 0.5% NAC (Exir Pharmaceutical Company, Tehran, Iran) dissolved in normal saline solution at a pH of 7.4 [18]. The right eyes of the Dox + CEES group were treated with 1 drop of purified liquid doxycycline (12.5 $\mu\text{g/ml}$) (Kimiadoxy 50^R, Iran) applied two times per day [19]. The right eyes of the rats of group 5 (Dox + NAC + CEES) were treated with 1 drop of doxycycline (12.5 $\mu\text{g/ml}$) two times per day, and 1 drop of 0.5% NAC 2 times per day separately to prevent the possible interaction between NAC and Dox. Right eyes were unexposed and were used as internal controls.

Rats were anesthetized by 35 mg/kg body weight, ketamine hydrochloride, and xylazine combination, and then, were sacrificed. The cornea and the aqueous humor were rapidly separated for the next experiments. From each group, three tissues were kept at 10% buffered formalin for histological examination, and three tissues were used for molecular and biochemical analysis, as described below.

Corneal opacity and neovascularization scoring

The degree of opacification was assessed during 21 days after CEES-exposed cornea according to the Oxford system [20]. Clinical assessments were also performed at 1, 3, 7, and 21 days to measure the haze degree and scored between 0 and 4 numerical scales. 0: clear cornea; 1: slight stromal opacity with pupil margin; 2: moderate stromal opacity with only pupil margin visible; 3: intense corneal opacity with difficulty anterior visible; and 4: maximal corneal opacity without the ability to view the anterior chamber.

Neovascularization initiated from dilated limbal was based on the extent of centripetal invasion of the vessels in the cornea regions. On the 3rd, 7th, and 21st days, the slit lamp biomicroscope was scored 0–3 for each corneal based on a similar study [21] (0: no neovessels; 1: neovessels at the corneal limbus; 2: neovessels spanning the corneal limbus and approaching the corneal center, and 3: neovessels spanning the corneal center). Vessel branches, which were not penetrated into the corneal stroma, had not been counted for neovascularization.

Histological analyses

The anterior part of the eye, including the cornea and aqueous humor (anterior chamber), was fixed in 10% buffered formalin for 24 h. Subsequently, samples were embedded in paraffin and cut into 5- μm thick. After deparaffinization, tissues were stained with hematoxylin and eosin (H&E) for histopathological examination, the thickness of the cornea, the numbers of polymorph nuclear cells infiltrated into the

cornea, and the intensity of neovascularization were measured by the digital image analyzer.

Real-time PCR

Three corneal tissues from each group were divided into two equal parts. One part was homogenized in a 1 ml RNX-Plus reagent (Cat. no. EX6101; SinaClon BioScience, Tehran, Iran) for extract RNA, and another part was used for biochemical experiments. The purification and quantity of total RNA were measured using a Thermo Scientific™ NanoDrop 2000 (Thermo Fisher Scientific Co., Waltham, MA) at 260/280–260/230 ratios, and then cDNA was synthesized with Revert Aid first-strand cDNA synthesis kit (YTA, Tehran, Iran) according to the manufacturer's instructions.

The real-time PCR was applied to indicate quantification of mRNA on a Rotor-Gene RG-300 (Corbett Research, Sydney, Australia) and SYBR Green Real-time PCR Master Mix Kit (TAKARA, Tokyo, Japan, catalog no. RR820Q). In detail, thermal cycling that was exploited in the process started with denaturation step at 95 °C for 5 min, and followed by 38 cycles of 95 °C for 15 s, 61 °C for 20 s, and 72 °C for 25 s. Relative expression levels of *tnf-α*, *nf-κB*, *cxcl-1*, *vegf* and *mmp2*, 9 genes were calculated using the ($2^{-\Delta\Delta C_t}$) method; and normalized versus expression of β -actin gene. The primer sequences are documented in [Supplementary data](#) files.

Biochemical examination

Three corneal tissues stored at –80 °C from each group were placed in the cold PBS (100 mM, pH 7.4), and then homogenized at 4000–6000 RPM. After that, supernatant was collected, and transfer to a clean tube to determine the catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase

(GPx) activities by calorimetrically enzymatic assay kits (ZellBio GmbH, Ulm, Germany). The sensitivity of these kits were 0.5 U/mg.

Statistical analysis

All data were normal and presented as mean ± SD. The two-way ANOVA was used to perform the statistical analysis of the results. The data of CEES group (group 2) were analyzed in comparison to other groups at 3, 7, and 21 days separately. Spearman correlation analysis was also used to estimate the correlations between gene expression. GraphPad Prism software version 6 (GraphPad Software, San Diego, CA) used for data analyses. The *p*-value was set < .05 as statistically significant.

Results

NAC treatment altered the antioxidant defense system of the cornea under CEES-induced damage

We first evaluated innate antioxidant defense consistent with the single and combined topical treatment of NAC and Dox in CEES-damage cornea. In comparison with CEES group (group 2), antioxidant enzymes activity of other groups was separately analyzed at 3, 7, and 21 days. As the CAT, SOD, and GPx activities were previously shown to be affected in sulfur mustard exposure [22]. The activity of all these enzymes significantly declined by three-fold on day 3 after the CEES damage in the CEES group compared to the healthy cases (*p*-value < .05). Furthermore, these enzyme activities remained low in the entire period of the experiment (Figure 1). It was noticeable that the CAT and SOD activity levels significantly increased to 6.775 and 77.395 U/mg on the 21st day of NAC+CEES treatment (group 4);

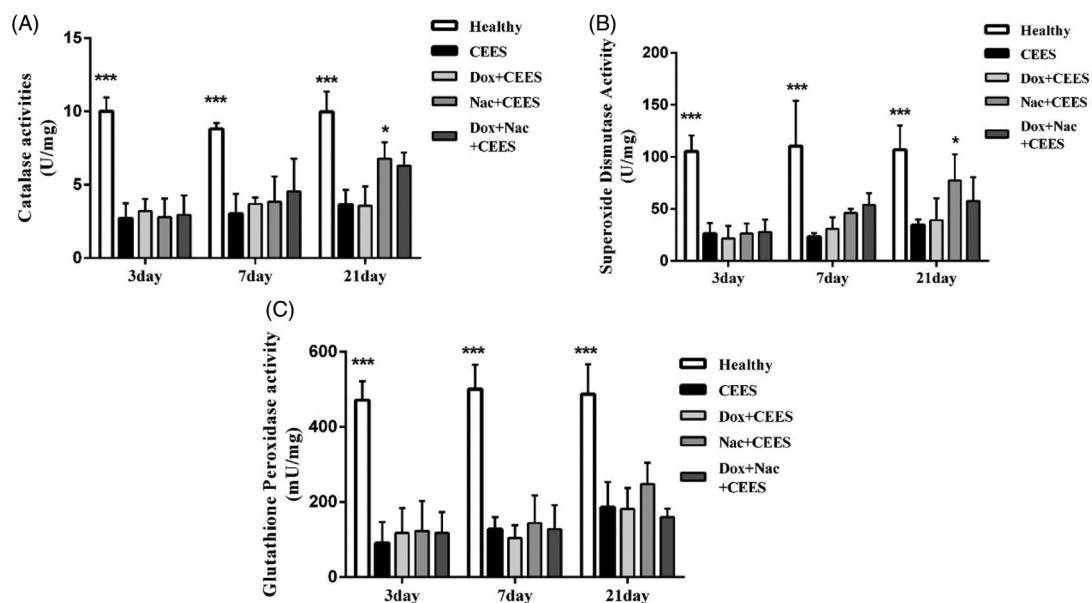


Figure 1. Antioxidant activity in the cornea during 3, 7, and 21 days time period after CEES injury. The CAT (A) and SOD activity (B) showed an increase in the NAC-treated group 21 days following CEES cornea damage (*p*-value < .05), but changes in the GPx were not statistically significant. Data were expressed as mean ± SD. Indicators *p*-value ≤ .05: *, *p*-value ≤ .01: **, *p*-value ≤ .001:*** compared with the CEES group at 3rd, 7th, and 21st days separately. CAT: catalase; SOD; superoxide dismutase; GPx: glutathione peroxidase; Nac: *N*-acetyl cysteine.

however, the enhancement of GPx activity in this group had no statistically significant (p -value $> .05$; Figure 1(C)). Although, GPx, CAT, and SOD activity of Dox+CEES and Dox+NAC+CEES groups (groups 3 and 5) continued a gradual rise at 7th and 21st days following CEES cornea damage, but were not fully recovered in the period of 21 days (p value $> .05$; Figure 1). Therefore, the NAC treatment revealed changes in antioxidant defense, particularly in the case of 21 day treatment.

NAC suppressed TNF- α -Rel-a pathway in CEES-injured cornea

We next evaluated the gene expression of the *tnf- α* in the CEES-injured cornea (group 2) by real-time PCR in comparison to other groups at 3rd, 7th, and 21st days separately. As it was reported, TNF- α increased in the injured cornea which was under oxidative stress [23]. Accordingly, we found a significant increase of *tnf- α* gene expression in the cornea of the CEES group (group 2) compared to the healthy group

(group 1) in all selected time points (p -value < 0.05 ; Figure 2(A)). Moreover, the mRNA level of TNF- α at 7th and 21st days of NAC+CEES and Dox+NAC+CEES groups (groups 4 and 5) decreased considerably (p -value $< .05$); however, the comparison of *tnf- α* gene expression at 3rd days between these groups did not show any statistically signification (Figure 2(A)).

As can be observed in Figure 2(B), the mRNA expression of *rel-a* also increased in the cornea of rats treated with CEES (group 2) than the healthy group at 7th and 21st days. Similar to *tnf- α* gene expression, the expression of *rel-a* gene declined following the 7th and 21st days of NAC and Dox+NAC topical treatment (p -value $< .05$; Figure 2(B)). According to the spearman correlation analysis, the mRNA levels of *tnf- α* in the CEES group positively correlated with that of in Rel-a CEES group (Table 1). Interestingly, this correlation was also seen in groups 4 and 5, which were treated with NAC and Dox+NAC (r : 0.863 and 0.730 respectively, Table 1). Therefore, the use of the NAC drop eye could attenuate the TNF- α -Rel-a pathway via reducing *tnf- α* and *rel-a* gene expression.

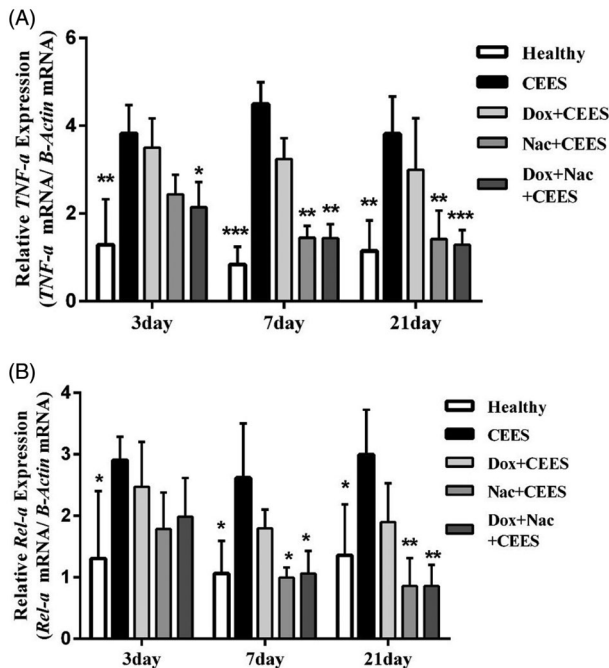


Figure 2. TNF- α and Rel-a mRNA level was evaluated in the CEES-injured cornea at the 3rd, 7th, and 21st days by real-time PCR. Compared with the CEES group, TNF- α (A) and Rel-a (B) mRNA level in NAC+CEES and Dox+NAC+CEES groups significantly decreased after 7 and 21 days (p -value $< .05$). Data were expressed as mean \pm SD. Indicators p -value $\leq .05$: *, p -value $\leq .01$: **, p -value $\leq .001$:*** compared with the CEES group at the 3rd, 7th, and 21st days separately. Nac: N-acetylcysteine; Doxy: doxycycline.

NAC and doxycycline could not decrease *cxcl-1* mRNA expression

Based on the Cxcl-1 evaluation carried out by RT-PCR, *cxcl-1* gene expression increased in the cornea of rats exposed to CEES (group 2) compared to healthy cases at 3rd days (p -value $< .05$; Figure 3(A)). The comparison of *cxcl-1* gene expression between group 2 and treatment groups at 3rd, 7th, and 21st days did not show any statistically significant data (p -value $> .05$). There was a slight decrease in the Cxcl-1 mRNA expression at 7 and 21 days after cornea damage with CEES in group 2. Surprisingly, a similar Cxcl-1 expression pattern was also shown in the all treatment groups (Figure 3(A)). Therefore, the treatment of NAC and Dox does not seem to affect *Cxcl-1* gene expression.

NAC and doxycycline down-regulated angiogenesis factors at 7th and 21st days

To determine the effects of NAC and Dox on the expression of angiogenesis factors, the mRNA level of VEGF-A, MMP-2, and MMP-9 was evaluated. The differential expression of *vegfa* gene in Figure 3(B) showed that the mean *vegfa* gene expression in group 2 seemed to be more than healthy samples. Similar to *rel-a* gene expression, mRNA level of VEGF-A reduced in CEES-injured rats treated with NAC and

Table 1. Spearman correlations between genes expression.

Gene	Group			
	CEES <i>r</i> (<i>p</i> -value)	Doxy + CEES <i>r</i> (<i>p</i> -value)	NAC + CEES <i>r</i> (<i>p</i> -value)	Dox + NAC + CEES <i>r</i> (<i>p</i> -value)
<i>tnf-α</i> & <i>rel-a</i>	0.6862 (.0475)*	0.3810 (.3599)	0.863 (.0058)*	0.7306 (.0467)*
<i>rel-a</i> & <i>cxcl-1</i>	-0.2594 (.4815)	0.3095 (.4618)	0.3333 (.4279)	0.5988 (.1237)
<i>rel-a</i> & <i>vegfa</i>	0.7113 (.0381)*	0.1071 (.8397)	0.7857 (.0279)*	0.4671 (.2478)
<i>rel-a</i> & <i>mmp2</i>	0.8144 (.0186)*	0.1429 (.7520)	0.3571 (.3894)	0.6826 (.0701)
<i>rel-a</i> & <i>mmp9</i>	0.4286 (.2482)	0.2619 (.5364)	-0.2619 (.5364)	0.4058 (.4333)

*The P -value was set as statistically significant.

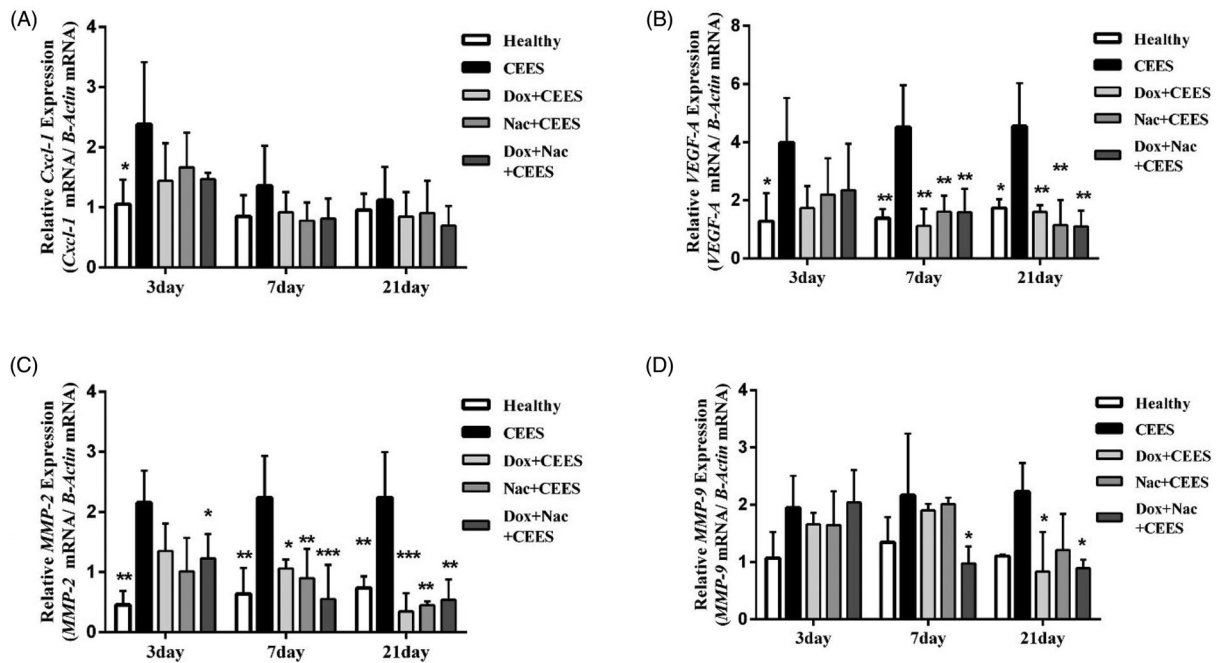


Figure 3. Cxcl-1, VEGF-A, and MMP2, 9 mRNA level was evaluated at the 3rd, 7th, and 21st days in the cornea of rats exposed to CEES. (A) The *cxcl-1* gene expression did not show any significant reduction in the treatment groups (p -value $> .05$). (B) VEGF-A mRNA level seemed to be under the effect of both NAC and Dox treatment. (C and D) The gene expression of *mmp2, 9* significantly declined after 21 days of Dox + NAC treatment. Data were expressed as mean \pm SD. Indicators p -value $\leq .05$: *, p -value $\leq .01$: **, p -value $\leq .001$: *** compared with the CEES group at the 3rd, 7th, and 21st days separately. Nac: N-acetyl cysteine; Doxy: doxycycline.

Dox + NAC (groups 4 and 5) at 7th and 21st days. In addition, spearman analysis indicated that *vegfa* gene expression was correlated with the *rela* gene expression in the NAC-treated group (r : 0.785; p -value: .0279; Table 1). Interestingly, Dox treatment (group 3) after 7th and 21st days statistically decreased the mRNA expression of VEGF-A (p -value $< .05$). The correlation analysis in Table 1 showed that there was no correlation between VEGF-A and *Rel-a* mRNA expression in the Dox treatment group. Thus, the VEGF-A mRNA level seemed to be under the effect of both NAC and Dox treatment; however, the mechanism of Dox effect on VEGF-A expression was not related to changes in *Rel-a* expression.

Figure 3(C,D) indicates that both MMP-2 and MMP-9 as the main functional enzymes in angiogenesis were overexpressed in the cornea injured with CEES rather than the healthy cases. In addition to NAC, Dox topical treatment and combined treatment with Dox + NAC could decrease gene expression of *mmp2, 9* at the 21st day (p -value $< .05$; Figure 3(C,D)). Notably, there was no correlation between *rel-a* and *mmp-2, mmp-9* gene expression in the treatment group, indicating that the reduction of MMP-2, MMP-9 mRNA expression may not be related to the decline in *Rel-a* expression (Table 1).

NAC clinically changed the infiltration of inflammatory cells and CNV

The effects of NAC and Dox treatments at 3rd, 7th, and 21st days were investigated by performing histological analyses using hematoxylin and eosin (H&E) staining. In the case of the CEES damage model at 3 and 7 days (during early phase), the infiltration of the inflammatory cells was detected in the

cornea, stroma, and anterior chambers. The edema of stroma and interrupted squamous epithelium were also noticeable in the CEES group (Figure 4). On the 3rd and 7th days, stroma exhibited a massive degree of apoptosis in corneal keratocytes. By the 21st day (late phase), dramatic neovascularization (shown by arrow) and collagen synthesis appeared at the cornea; however, edema was disappeared and the rupture epithelialization process occurred (Figure 4). Corneal neovascularization scoring indicated that there were significant increases in CNV scores in the CEES group from the 7th day to the 21st day after CEES injury (Figure 5). There was no obvious differentiation between corneas of the CEES group, but NAC + CEES and Dox + NAC + CEES groups after 7 days displayed a remodeling stromal and slightly recovered epithelium.

Also, the number of inflammatory cells between the Bowman membrane and Descemet's membrane was lesser compared to the CEES cases. Post 21 day treatment with NAC, a thin epithelium layer appeared over the surface of the cornea (Figure 4). The greatest inhibitory effect on CNV was detected in the combined treatment of Dox + NAC drop eye during 21 days post-injury. The neovascularization derived from limbus had a significant decline rather than the CEES group. The CNV score in the Dox + NAC + CEES group (group 5) at 7th and 21st days were 1.4 and 0.8, respectively (Figure 5), but the existence of a low number of macrophage cell (MQ) was still seen in the 21st day of groups 3 and 5. It was noticeable that the neovascularization score at the 21st day after Dox treatment decreased similar to 21st day Dox + CEES and Dox + NAC + CEES groups. In contrast, the presence of inflammatory cells infiltrated to stroma was significant 7 and 21 days following doxycycline treatment

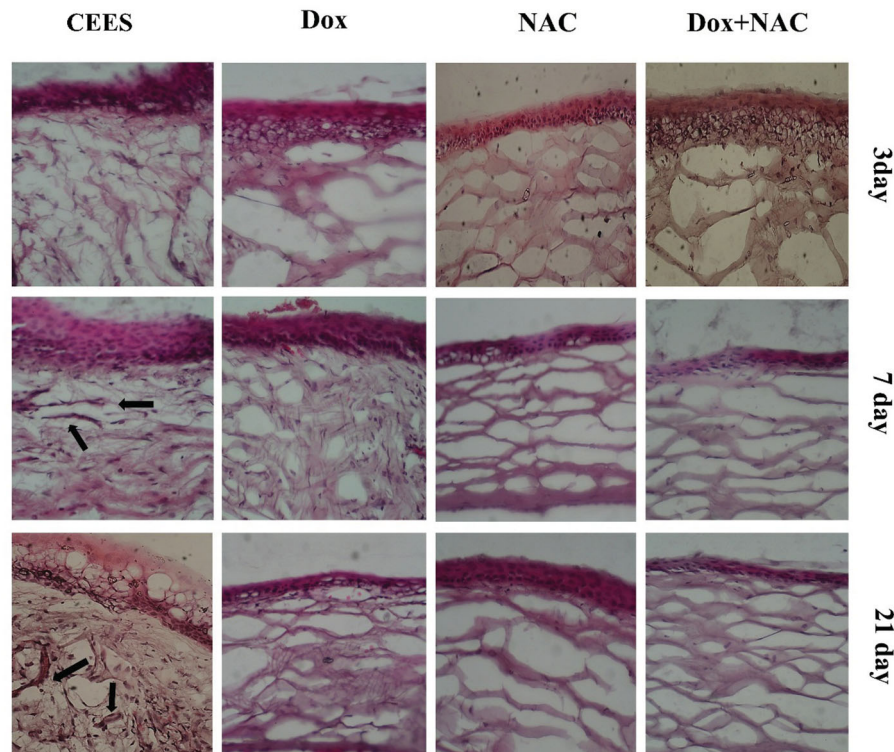


Figure 4. Effects of different treatments in H&E staining of corneal tissues after CEES injury. The infiltration of the inflammatory cells was seen in the cornea and stroma in the CEES group at the 3rd and 7th days. On the delay phase (21st day) dramatic neovascularization appeared in the CEES group (shown by arrow). NAC and Dox + NAC treatments after 7 days displayed a remodeling stromal and slightly recovered epithelium. The neovascularization score on the 21st day of treatment groups decreased; however, the inflammatory cells infiltrated to stroma still existed.

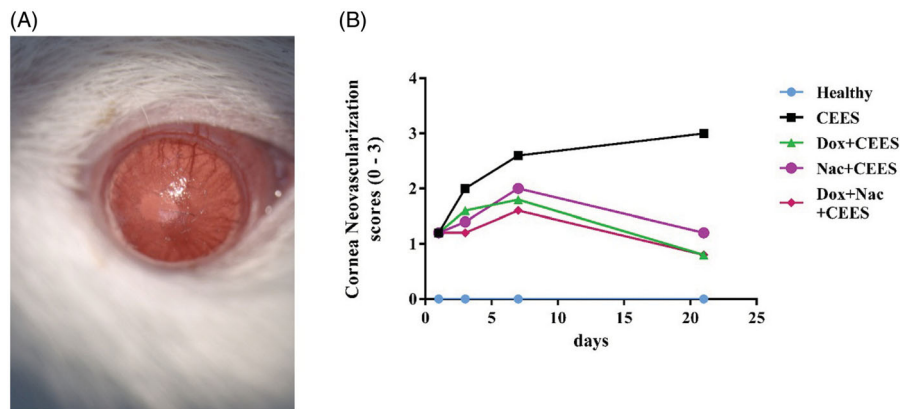


Figure 5. Effects of different treatments on CNV scores after CEES injury. (A) CNV on the 21st day after CEES cornea injury. (B) Less corneal neovascularization was observed by the time rats were treated with Dox and Dox + NAC at the 7th day (p -value $\leq .05$) and the 21st day (p -value $\leq .001$) rather than the CEES group. The CNV in the NAC + CEES group at the 21st day was lower than CEES group (p -value $\leq .01$). The mean clinical CNV score indicated that the 21st day post Dox treatment had a significant decrease in the score at about 1.2.

(Figure 4). Thus, Dox eye drop may provide a therapeutic agent for inflammatory and angiogenic factors.

The corneal opacity score was lower in both Dox + CEES and Dox + NAC + CEES groups than in the CEES group (score: 4) 7 and 21 days after CEES injury (Supplementary Figure 2). Also, the clinical evaluation indicated that Dox treatment yielded similarly lower corneal opacity scores ranging from 1.5 to 2 on the 21st day (Figure 6).

Discussion

Vesicating agent SM causes injuries in ocular tissue at much lower concentration rather than other organs. Corneal

neovascularization associated with SM is considered a challenge in terms of both intracellular pathways and clinical aspects. Despite numerous investigations, effective therapy to reverse the devastating mechanisms resulting in ocular injury has not been found [5]. In this study, we investigated single and combined treatments of NAC and Dox to treat the rat model of SM-induced cornea with delayed CNV.

To study ocular injury induced in the rat model, CEES was used in our study as a surrogate substance for SM. CEES is a mono alkylating agent modifying cellular macromolecules through an increase of ROS production [24]. Innate antioxidant defense in ocular tissues converting ROS to less reactive species. SOD acts as a disproportionation of superoxide

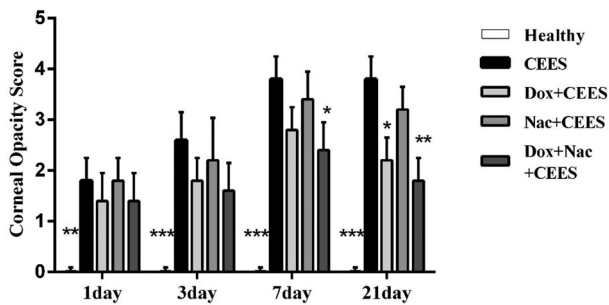


Figure 6. Effects of different treatments on cornea opacity scores after CEES injury. On the 21st day, the corneal opacity score was lower in both Dox + CEES and Dox + NAC + CEES groups, at about 2.5 and 2 respectively. Indicators p -value $\leq .05$: *, p -value $\leq .01$: **, p -value $\leq .001$: *** compared with the CEES group at the 1st, 3rd, 7th, and 21st days separately. Nac: N-acetyl cysteine; Doxy: doxycycline.

anion into hydrogen peroxide and molecular oxygen, while CAT and GPx catalyze the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. *In vitro* and *in vivo* vesicant-induced oxidative stress in ocular tissues demonstrated the reduction of catalytic antioxidant activity such as CAT, SOD, and stored glutathione (GSH + GSSG) [22]. We evaluated the activity of three antioxidant defense enzymes including CAT, GPx, and SOD. The low enzyme activity of CAT, GPx, and SOD was observed after 3 days CEES exposure when the greatest apoptotic corneal keratinocytes and epithelium degradation were detected using histological assessments. The CAT and SOD activities were increased by NAC and Dox + NAC treatment at the 21st day. It could be explained by the antioxidant capacity of NAC to detoxicate the electrophilic xenobiotics and modulate the redox signal transduction [25]. Similar enhancement of antioxidant enzyme activity was observed in the management of retinal oxidative stress by NAC [26]. Amir et al. did not announce any changes in glutathione of aqueous humor 24 h post-2,2-dichlorodiethyl sulfide (HD) exposure [27]. Our data of enzyme activities did not show any positive response to Dox treatment in all periods. Although NAC eye drop could not completely restore the activity of antioxidant enzymes in general, it promoted cell survival and cell recovery through ROS elimination.

ROS accumulation to some extent can be a result of the CEES agents that recruit and activate neutrophils and macrophages to induce inflammatory cytokines and signaling pathways. We explored the effects of NAC and Dox on *tnf- α* and *rel-a* gene expression, as the main inflammatory pathway. The expression level of *TNF- α* increased in the cornea of the CEES group, which represented the high level of oxidative stress, rather than healthy rats. Our data showed that NAC could decrease the *TNF- α* mRNA expression, probably through the cellular redox regulation. This alteration was also illustrated in the reduction of *rel-a* gene expression following NAC treatment in the long term. *TNF- α* is one of the pro-inflammatories elevated in the cornea of BALB/c male mice with alkali corneal burns. The injection of infliximab, an anti-*TNF- α* antibody, was able to reduce inflammatory cytokines such as IL-1 β and IL-6 [28]. *TNF- α* cytokine potentially recruits neutrophil and macrophage (MQ) cells into the injured cornea *via* inducing the Rel-a to form nuclear factor kappa B (NF- κ B) transcription factor [29,30]. Correlation

analysis indicated the suppression effect of NAC on inflammatory signals between *TNF- α* and Rel-a.

The result of histological assessment exhibited a massive infiltration of inflammatory cells in the cornea exposed to CEES in the whole period of our experiment. Although NAC slightly declined the number of infiltrated neutrophils in the stroma of cornea, the number of macrophages was still high compared to the healthy cornea. Cxcl-1 is known as the main chemo-attractive factor for neutrophils in inflamed tissues. It has been reported that murine alkali-burned corneas associated with dry eye had a high level of Cxcl-1 expression, and dexamethasone treatment decreased both Cxcl-1 expression and infiltration on neutrophils [14]. The over-expression of *cxcl-1* gene was detected overall days of groups, and its down-regulation also occurred in the following days. Likewise, there was no relationship between Rel-a and Cxcl-1 expression. It was suggested that Cxcl-1 chemokine expression was not under the effect of Rel-a, as the main transcription factor, and other inflammatory signals may affect Cxcl-1 expression. Lu et al. reported that alkali-injured mice with *TNF* receptor 1-deficient (*TNF-Rp55* KO) suppressed expression of angiogenesis factors such as VEGF and nitric oxide synthases (iNOS), but intracorneal infiltration of leukocytes did not change, probably because it had few effects on the expression of a sustained CCL-2 expression [31].

Inflammation and downstream signals derived from immune cells after cornea injury trigger neovascularization with the involvement of angiogenesis factors and extracellular matrix (ECM) degradation [30]. In regard to the histological and clinical evaluations, Dox + NAC had significant effects on CNV inhibition on the 21-day CEES-induced injury group. The combined treatment Dox + NAC at the 21st day resulted in the lowest CNV and opacity score. In terms of angiogenesis factor expression, VEGF-A mRNA expression declined in the treatment group, especially in the Dox + NAC + CEES group. The reduction of VEGF-A expression in the NAC + CEES group was correlated with the low expression of Rel-a. Activated NF- κ B transcription factor has been shown to contribute to the production of VEGF-A that plays the main role in endothelial cell proliferation and CNV [32]. Solely doxycycline eye drop has also been proved to decrease CNV in various mechanisms. Wenru Su et al. in 2013 showed that doxycycline can inhibit human umbilical vein endothelial cells (HUVEC) proliferation by attenuating phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway [33]. A study reported that doxycycline can function as an inhibitor on tyrosine-phosphorylated VEGF-R1 and VEGF-R2. In addition, doxycycline by reducing the activity of matrix metalloproteinases (MMP2,9) activity and their expression can stop VEGF-A chamber release [34]. In our study, NAC and doxycycline declined both MMP-2 and -9 mRNA expression at the 21st day after treatment. It has been proved that doxycycline can control *mmp-2*, 9 gene expression by attenuating the PI3K/Akt pathway [33]. MMP2, 9 belong to the family of zinc-dependent endopeptidases that are activated by *TNF- α* in neutrophil and macrophages [6]. MMP2, 9 have the ability to break down the tight junction as well as collagen IV in the basement membranes of

endothelial [35]. In the alkali injury of the cornea, MMP-9 is responsible for the degradation of the basement membrane and tight junction between epithelial cells [16]. Our H&E staining showed that the existence of interrupted squamous epithelium was significant in the cornea injured with CEES; however, it was improved in the long run treatment. The process of healing of the injured cornea is complex, especially when ECM is remodeled, or the migration of inflammatory and endothelium cells is not controlled, thus, further studies are needed to remove the veil of ignorance. However, the limitation of our study was to analyze inflammatory and angiogenesis factors just at the level of mRNA expression. It is suggested that the protein expression of TNF- α , Rel-a, Cxcl-1, VEGF-A, and also, the enzyme activity of MMP-2,9 will be measured to shed a light on the effects of NAC and doxycycline on the model of SM-injured cornea.

Conclusion

The results indicated that the consequence of progressive inflammation in the CEES-injured cornea of rats led to the induction of neoangiogenesis. We found the beneficial effects of NAC and Dox treatment in decreasing developed CNV and angiogenesis factors. Despite the decline of inflammatory signals, these treatments could not completely prevent the infiltration of inflammatory cells. More research needs to have better insight into the recruitment pattern of macrophages.

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Disclosure statement

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Data availability statement

Data are available if requested.

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